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Synthesis of a Magnosalin Derivative, 4-(3,4,5-Trimethoxyphenyl)-6-(2,4,5-trimethoxyphenyl)-2-diethylaminopyrimidine, and the Anti-Angiogenic and Anti-Rheumatic Effect on Mice by Oral Administration

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Abstract—We describe here the synthesis and the anti-angiogenic and anti-rheumatic activities of 4-(3,4,5-trimethoxyphenyl)-6-(2,4,5-trimethoxyphenyl)-2-diethylaminopyrimidine (TAS-202), a derivative of magnosalin, which is a natural product isolated from *Flos magnoliae*. TAS-202 inhibited the proliferation of vascular endothelial cells more potently than magnosalin, and when given orally it inhibited basic fibroblast growth factor (bFGF)-induced angiogenesis and collagen-induced arthritis in mice. This magnosalin derivative with anti-angiogenic effects is a candidate for the treatment of rheumatoid arthritis.

Rheumatoid arthritis (RA) is a complex chronic inflammatory disease, and angiogenesis is now recognized as an essential component of chronic inflammation such as pannus development in RA.[1–4] In addition, the increased vascular endothelial cells function as a rich source of inflammatory cytokines.[5] Therefore, the inhibitory effects against the proliferation of vascular endothelial cells on inflammatory sites lead to limited pannus growth and joint destruction in RA.

'Shin-i' (*Flos magnoliae*) is the dried flower buds of *Magnolia salicifolia* Maxim and has been used traditionally in the treatment of chronic nasal inflammation.[6] Magnosalin and magnoshinin (Fig. 1) are neolignans isolated from 'Shin-i'.[7] The anti-chronic inflammatory effect of 'Shin-i' is caused by the selective inhibition of angiogenesis by magnosalin and of granuloma formation by magnoshinin.[6] Magnosalin also inhibits the proliferation of vascular endothelial cells.[8] Thus, magnosalin seems to exhibit anti-rheumatic effects based on anti-angiogenic action in mice if given intraperitoneally.

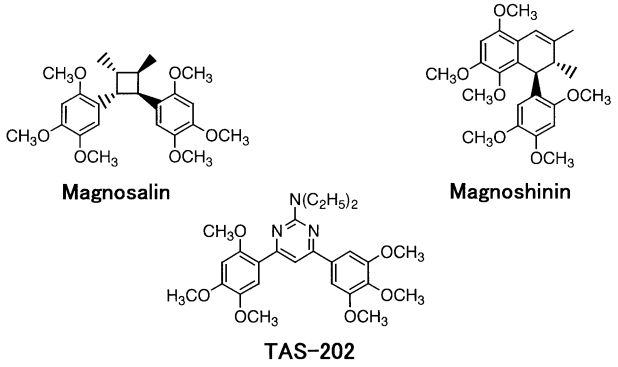

Figure 1. Chemical structures of magnosalin, magnoshinin and a derivative, 4-(3,4,5-trimethoxyphenyl)-6-(2,4,5-trimethoxyphenyl)-2-diethylaminopyrimidine.

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Since we noted the inhibitory effects of magnosalin on the proliferation of endothelial cells, a series of magnosalin derivatives were synthesized and we evaluated their anti-proliferating effects in vitro and anti-inflammatory effects by oral administration in vivo. The most potent compound of this series was 4-(3,4,5-trimethoxyphenyl)-6-(2,4,5-trimethoxyphenyl)-2-diethylaminopyrimidine (TAS-202) (Fig. 1).

Synthesis of TAS-202

TAS-202 was synthesized in four steps, starting from 2,4,5-trimethoxybenzaldehyde **1** as shown in Scheme 1.[9,10] **2** was synthesized by reacting **1** with the complex which was prepared from triphenylphosphine and carbon tetrabromide. **3** was prepared by lithiation of **2**, followed by treatment with 3,4,5-trimethoxybenzaldehyde, and oxidation using activated MnO_2. Finally, TAS-202 was obtained by cyclization of **3** with 1,1-diethylguanidine in 45% overall yield.

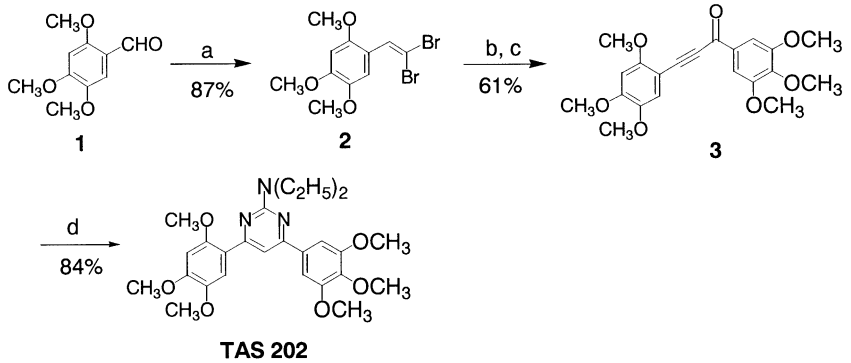
Inhibitory effect of TAS-202 on the proliferation of human endothelial cells, fibroblasts and cancer cells

Magnosalin, magnoshinin and a derivative, TAS-202, were tested for their inhibitory effects on the proliferation of human umbilical vein endothelial cells (HUVEC) in response to endothelial cell growth supplement (ECGS). The 50% inhibitory concentrations of magnosalin and magnoshinin were 1.11 μM (95% confidence limit: 0.64–1.71) and 7.67 μM (6.08–9.45), respectively. TAS-202 was 7.5 and 51.8 times more potent than magnosalin and magnoshinin, respectively. The effects of TAS-202 on HUVEC, normal human dermal fibroblasts (NHDF), and KB cells (a head and neck

Table 1. Anti-proliferative effects of TAS-202 on cultured human endothelial cells, fibroblasts and cancer cells

Cell	Inducer	TAS-202 (μM)	[^{3}H]-Thymidine incorporation (cpm)[a]	Inhibition (%)[b]	IC_{50} (μM)	95% Confidence limit
HUVEC	ECGS	0	2810.3±54.0			
		0.1	1634.5±78.3	41.8		
		0.3	994.2±73.2	64.6	0.148	0.117–0.179
		1	359.0±30.4	87.2		
	VEGF	0	2077.3±175.0			
		0.1	1861.3±142.5	10.4		
		0.3	1263.7±38.8	39.2	0.398	0.321–0.508
		1	448.0±66.8	78.4		
	bFGF	0	9998.4±282.4			
		0.1	6638.9±937.1	33.6		
		0.3	6927.6±691.6	30.7	0.561	0.425–0.700
		1	2969.9±123.5	70.3		
	FBS	0	595.9±67.2			
		0.1	437.0±83.5	26.7		
		0.3	154.2±8.3	74.1	0.199	0.077–0.340
		1	122.8±25.9	79.4		
NHDF	FBS	0	1867.1±59.3			
		0.1	1907.7±25.2	−2.2		
		0.3	2209.9±82.0	−18.4	—	
		1	2133.2±160.3	−14.3		
KB cell	FBS	0	18896.0±720.6			
		0.1	18961.2±1387.5	−0.3		
		0.3	15784.2±848.7	16.5	—	
		1	13366.5±1648.2	29.3		

HUVEC, Human umbilical vein endothelial cells; NHDF, normal human dermal fibroblasts; KB cell, head and neck cancer cell line; ECGS, endothelial cell growth supplement; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; FBS, fetal bovine serum.
[a]Mean±SEM of triplicate.
[b]Inhibition % = (1−(agent/control))∗100.



Scheme 1. Reagents and conditions: (a) CBr_4, PPh_3, CH_2Cl_2; (b) (i) n-BuLi, THF, (ii) 3,4,5-trimethoxybenzaldehyde; (c) MnO_2, $CHCl_3$; (d) $(C_2H_5)_2NC(=NH)NH_2$, DHF.

cancer cell line) are summarized in Table 1. TAS-202 also inhibited the proliferation of HUVEC stimulated with a potent angiogenesis-inducer, vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF), or fetal bovine serum (FBS) alone. On the other hand, TAS-202 up to 1 μM showed no or little effect on the proliferation of NHDF or KB cells stimulated with FBS. These results show that TAS-202 may be a selective inhibitor of HUVEC proliferation.

Inhibitory effect of TAS-202 on angiogenesis in mice

To determine whether TAS-202 has an inhibitory effect on angiogenesis in vivo, an angiogenesis assay was carried out in mice. Mice were implanted with a gelatin sponge inserted with a pellet containing bFGF, and then received an oral administration of TAS-202 or prednisolone for 7 days. The sponge was removed and measured for hemoglobin (Hb) content. The sponge from control animals contained a significant amount of Hb (Fig. 2), while no increase in Hb content was observed in the sponge without bFGF (data not shown). TAS-202 (10 and 30 mg/kg/day) produced a dose-dependent inhibition of the increase of Hb content and prednisolone (10 mg/kg/day), which is a synthetic glucocorticoid, showed almost complete inhibition. The increase of Hb content in the sponge has been reported by Pesenti et al.[11] to be the parameter of vascularization in the model, showing that oral TAS-202 as well as prednisolone inhibits bFGF-induced angiogenesis.

Anti-rheumatic effect of TAS-202

We further examined whether an anti-angiogenic agent shows inhibitory effects on collagen-induced arthritis in mice. Arthritic symptoms were apparent 7 days after a booster injection and gradually increased until at least day 14 (Fig. 3). TAS-202 at 30 mg/kg/day given orally significantly inhibited the arthritic symptoms on days 12–14 (32.4% inhibition on day 14). Indomethacin at 1 mg/kg/day given orally also showed inhibitory effects on the arthritic score (22.2% inhibition on day 14). Interestingly, the combined treatment of TAS-202 (30 mg/kg/day) and indomethacin (1 mg/kg/day) resulted in a marked suppression of arthritic symptoms (75.0% inhibition on day 14). The results show that TAS-202 and IND prevented the arthritic response in a synergistic manner, indicating that the angiogenesis inhibitor together with non-steroidal anti-inflammatory drug (NSAID) may be of great benefit to RA therapy.

In conclusion, this magnosalin derivative with anti-angiogenic effects is a candidate for the treatment of rheumatoid arthritis.

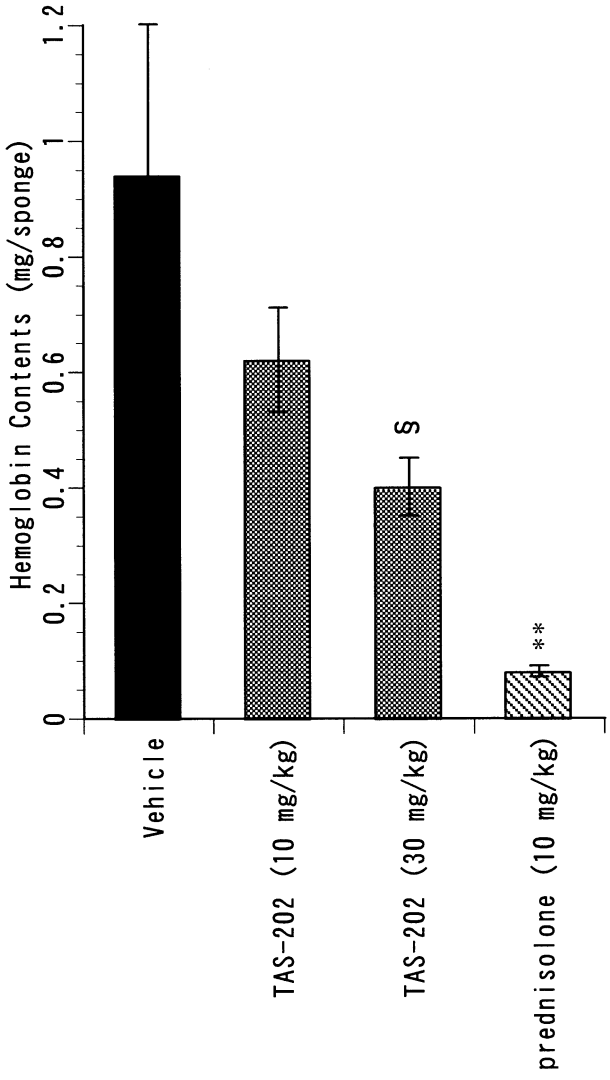


Figure 2. Inhibitory effects of TAS-202 on gelatin sponge-angiogenesis induced by bFGF in mice. Each agent was administered orally for 7 days from day 0. On day 7, the sponges were removed from the mice. Hemoglobin was extracted from the sponge and measured. Values are means ± SEM of 12 mice. $^{\S}p < 0.05$, $^{\S\S}p < 0.01$ versus vehicle (Dunnett's test). $^{*}p < 0.05$, $^{**}p < 0.01$ versus vehicle (Welch's *t*-test).

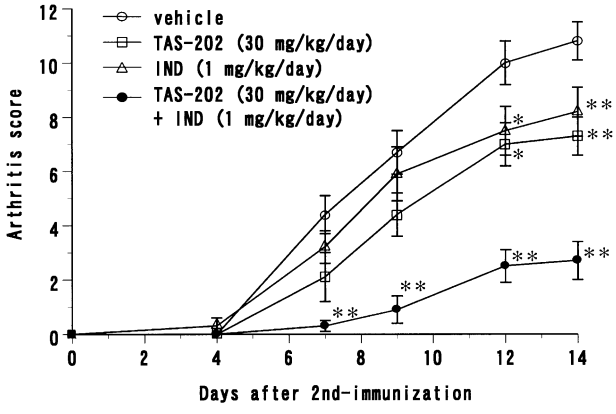


Figure 3. Inhibitory effects of TAS-202 and indomethacin (IND) on collagen-induced arthritis in mice. Each agent was administered orally for 14 days after the second immunization. Values are mean ± SEM of 10 mice. $^{*}p < 0.05$, $^{**}p < 0.01$ versus vehicle (Mann–Whitney *U*-test).

Biological Test Methods

Animals

Male C57BL/6j mice (6 weeks old) were purchased from Clea Japan Inc. (Tokyo, Japan). Male DBA1/j mice (10

weeks old) were purchased from Charles River Japan Inc. (Yokohama, Japan).

Proliferation assay

HUVEC purchased from Sankou Junyaku (Tokyo, Japan) were suspended in RPMI-1640 medium (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) supplemented with 2% FBS, 100 U/mL of penicillin, 100 mg/mL of streptomycin and an inducer: 10 μg/mL of ECGS (including 3 ng/mL of epidermal growth factor) or 10 ng/mL of bFGF or 10 ng/mL of VEGF. Three thousand cells were seeded in each well of 96-well flat plates with or without test compounds, including 0.3% dimethylsulfoxide (Nacalai Tesque, Kyoto, Japan). NHDF (Kurabo, Osaka, Japan) and KB cells (American Type Culture Collection, Rockville, MD, USA) were cultured in modified MCDB-202 medium (F-BM medium; Kurabo, Osaka, Japan) with 20% FBS and Eagle's minimal essential medium (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) with 10% FBS, respectively. Concentrations of growth factors including FBS were determined by preliminary experiments to give submaximum proliferation responses. They were cultured for 96 h, and the cultures were pulsed with 3.7 kBq of [^{3}H]-thymidine for the last 17 h of culture, followed by harvest on glass filter and washing with phosphate buffered saline, 5% trichloroacetate and ethanol. The uptake of [^{3}H]-thymidine was counted by Betaplate (Pharmacia LKB Biotechnology, Uppsala, Sweden) and expressed as cpm.

Gelatin sponge-angiogenesis assay

The assay was carried out according to the method of Pesenti et al.[11] with minor modifications. Briefly, pellets containing bFGF were prepared by mixing an equivalent volume of bFGF dissolved in 400 mg/mL of aluminum sucrose octasulfate (Sucralfate; Nippon Gouseikagaku Kogyo Co., Osaka, Japan) and poly(2-hydroxyethyl methacrylate) (Hydron® polymer Type Ncc; Polysciences Inc., Warrington, PA, USA) dissolved in ethanol.[12] After drying, the pellet was placed in the center of a piece of gelatin sponge (Gelfoam®; Nippon Upjohn, Tokyo, Japan). The sponge containing bFGF or solvent alone was subcutaneously implanted into the abdomen of C57BL/6j mice (one piece/one mouse). Seven days after implantation, the mice were sacrificed, and the sponge was carefully removed. Hemoglobin content (Hb) was extracted from the sponges in 0.1 M ammonia solution, and measured using a commercial assay kit (Hemoglobin B Testwako®, Wako Pure Chemical Industries Ltd, Osaka, Japan). Test compounds were administered orally from the day of implantation for 7 days.

Collagen-induced arthritis in mice

Male DBA1/j mice were intradermally injected with 0.1 mL of complete Freund's adjuvant containing 2 mg/mL of bovine type II collagen (Collagen Technical Service, Cosmo Bio, Tokyo, Japan). After 21 days, the same solution was injected intradermally to the base of the tail. Test compounds were given orally for 14 days after the booster injection. The severity of arthritis was evaluated by scoring each paw with an integer from 0 to 4 based on increasing erythema and swelling (0: normal; 16: maximum).[13]

Test compounds and reagents

Magnosalin and magnoshinin were isolated from magnolia by Professor Tohru Kikuchi in the Institute of Natural Medicine (Oriental Medicines) of our university.[7] They were dissolved in dimethylsulfoxide (Nacalai Tesque, Kyoto, Japan) for in vitro use. For in vivo administration, TAS-202, prednisolone (Nacalai Tesque, Kyoto, Japan) and indomethacin (Sigma Chemical Co., St. Louis, MO, USA) were suspended in soy bean oil. Fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), ECGS (Becton Dickinson Labware, Bedford, MO, USA), EGF and bFGF (Genzyme Co., Cambridge, MA, USA), VEGF (R&D Systems, Inc., Minneapolis, MN, USA) were also purchased.

Statistics

Data were expressed as the means ± SEM. Differences between the groups were evaluated by Dunnett's test, Welch's *t*-test or Mann–Whitney *U*-test when indicated. *p* Values less than 0.05 were considered significant.

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9. Characterization of TAS-202: mp 110–112 °C; ^{1}H NMR ($CDCl_3$, ref $CHCl_3$ = 7.27 ppm) δ 1.29 (t, J = 7.0 Hz, 6H, CH_3), 3.77 (q, J = 7.0 Hz, 4H, CH_2), 3.90 (s, 3H, CH_3O), 3.91 (s, 3H, CH_3O), 3.92 (s, 3H, CH_3O), 3.95 (s, 9H, CH_3O), 6.61 (s, 1H, Ar–H), 7.37 (s, 2H, Ar–H), 7.61 (s, 1H, Ar–H), 7.80 (s, 1H, Ar–H); anal. ($C_{26}H_{33}N_3O_6$) C, H, N.

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